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HPLC Method Development

- Strategy for Method Development
- HPLC Technique

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Strategy for Method Development

1. Define method and separation goals
2. Gather sample and analyte information
3. Initial method development (scouting runs)
4. Method fine-tuning and optimization

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1.Define method and separation goals

Method types

- Qualitative method : identification by matching the retention time with reference standard
- Quantitative method : Determine amount of analyte (s) in sample, require system calibration typically using external standard (s)
- Preparative method : isolate purified components in the sample

Method goals

- Sensitivity
- Efficiency
- Speed
- Ease of use
- Cost

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2.Gathering sample and analyte information

- Number of components and concentration range of analytes
- Chemical properties of analytes (structure, Mw, functional group, pKa, solubility)
- Sample preparation requirements
  - Filtration, centrifugation
  - Extraction
  - Derivatization (pre-column derivatization)
  - Solid handling (grinding, homogenization)

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3.Initial HPLC method development

3.1 Initial Detector selection

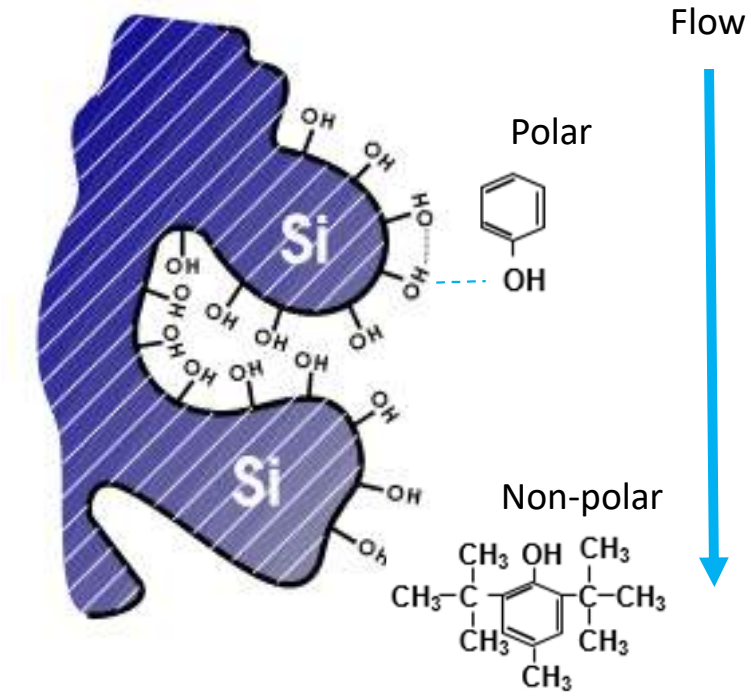
- UV/Vis absorbance : for chromophoric analytes
- Photo diode array (PDA) : same as UV/Vis also provides UV spectra
- Refractive index (RI) : for nonchromophoric analytes
- Mass spectrometer : for ionizable analytes more sensitive and specific

3.Initial HPLC method development

3.2 Selection of chromatographic mode

Normal phase chromatography (NPC)

- The separation mechanism based on polarity of functional group
- Usually non-aqueous , non-polar solvent
- Least commonly used



3.Initial HPLC method development

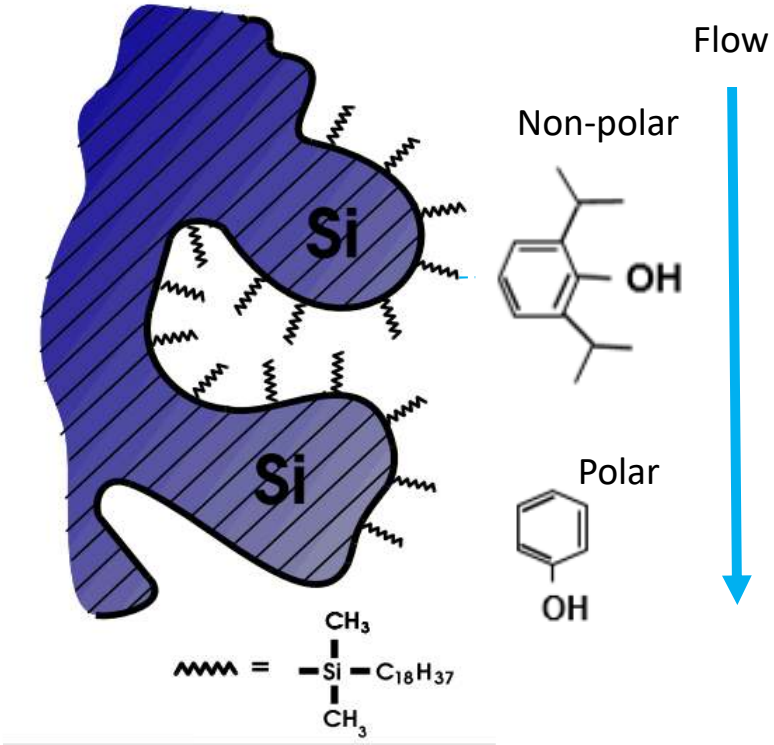
3.2 Selection of chromatographic mode

Reverse phase chromatography (RPC)

- The separation mechanism based

on hydrophobicity

- Usually aqueous , polar solvent
- The first choice for most regular samples



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3.Initial HPLC method development

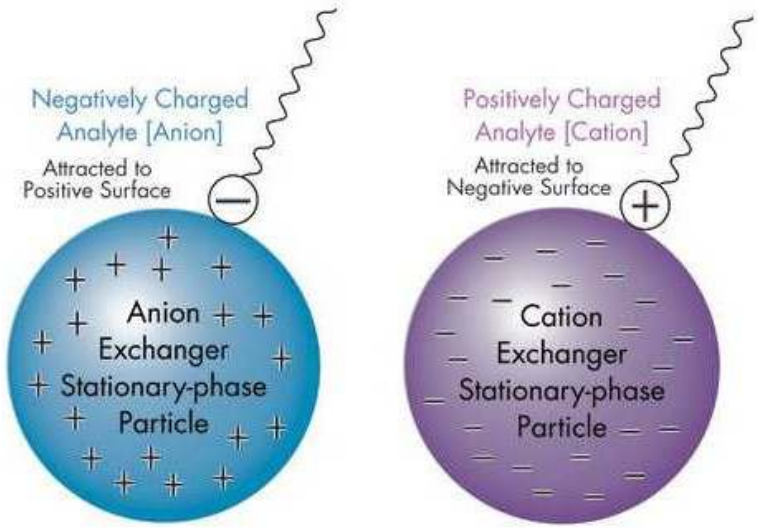
3.2 Selection of chromatographic mode

Ion-Exchange chromatography (IEC)

- The separation mechanism based

on the formation of transient ion bonds between charged samples and the oppositely charged column surface

- Resin-based ,anionic or cationic surface

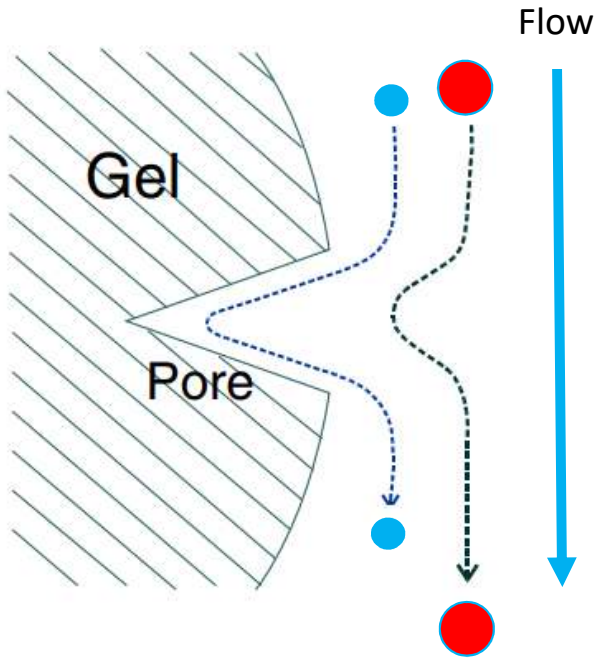


3.Initial HPLC method development

3.2 Selection of chromatographic mode

Size-Exclusion chromatography (SEC)

- The basis of separation is that molecules above a certain size are totally excluded from the pores and the interior of pores is accessible to smaller molecules
- Commonly used in polymer analysis to determine molecular weights



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3.Initial HPLC method development

3.3 Initial selection of HPLC column

- Types based on chromatographic mode :

(NPC, RPC, IEC, SEC)

- Types based on column dimension :

(column length, column inner diameter)

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4.Method fine-tuning and optimization

4.1 Mobile phase parameters

% Strong solvent, pH, Buffer concentration, Solvent type

4.2 Operating parameters

Flow rate, Column temperature, Gradient segment

4.3 Column parameters

Bonded phase type, Column dimension (length, id, particle size)

4.4 Detector setting

Wavelength, sampling rate, filter

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Reverse Phase Chromatography (RPC)

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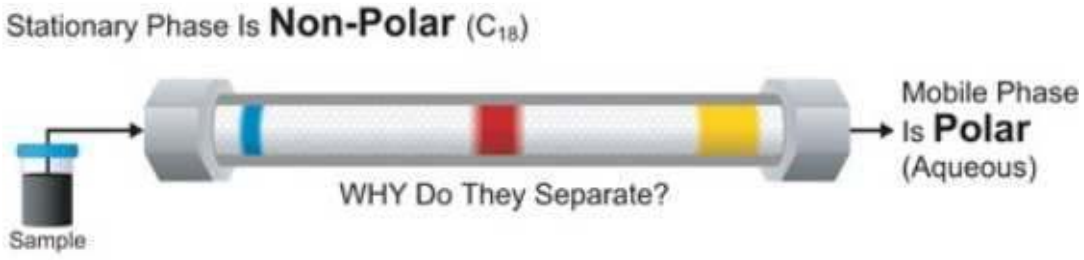
Reverse Phase Chromatography (RPC)

- The term reversed-phase describes the chromatography mode that is just the opposite of normal phase
- use of a polar mobile phase and a non-polar [hydrophobic] stationary phase



"like dissolves like" or "like adsorbs like"

Separation Mode	Stationary Ohase (particle)	Mobile Phase (solvent)
Normal Phase	Polar	Non-polar
Reverse Phase	Non-polar	Polar

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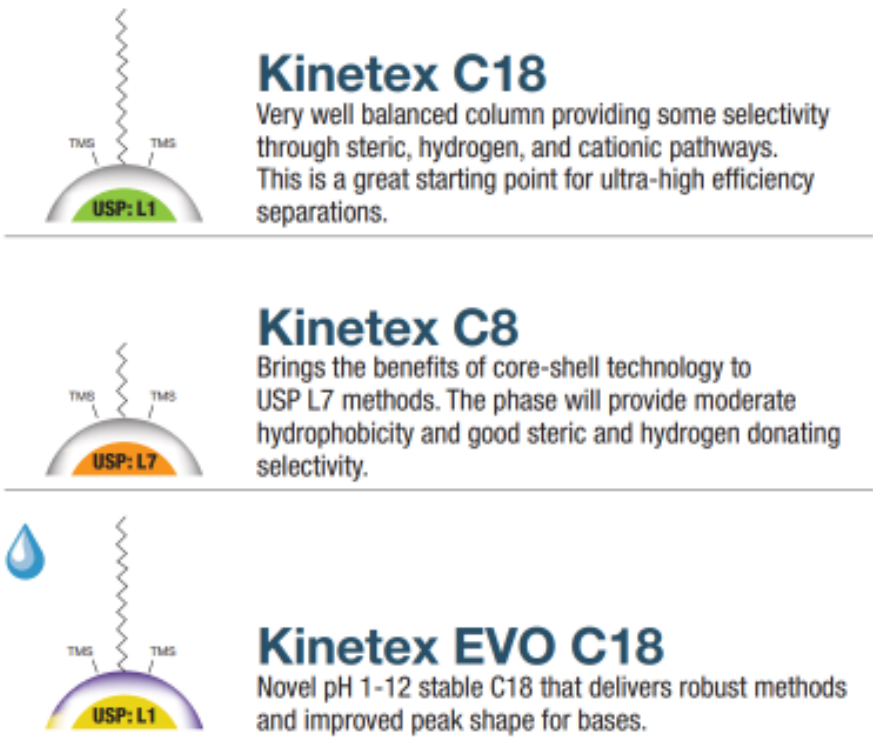
Surface of a Reversed-Phase Packing

Material characteristics

- Bonded phase
  - C8, C18, Phenyl
- Particle size (um)
  - 1.6,2.6, 3.5, 5
- Pore size (^{0}A)
  - 100, 200,
- Carbon load (%)
  - 9, 10, 11
- pH stability
  - 1.5-8.5, 1-12
- Pressure stability



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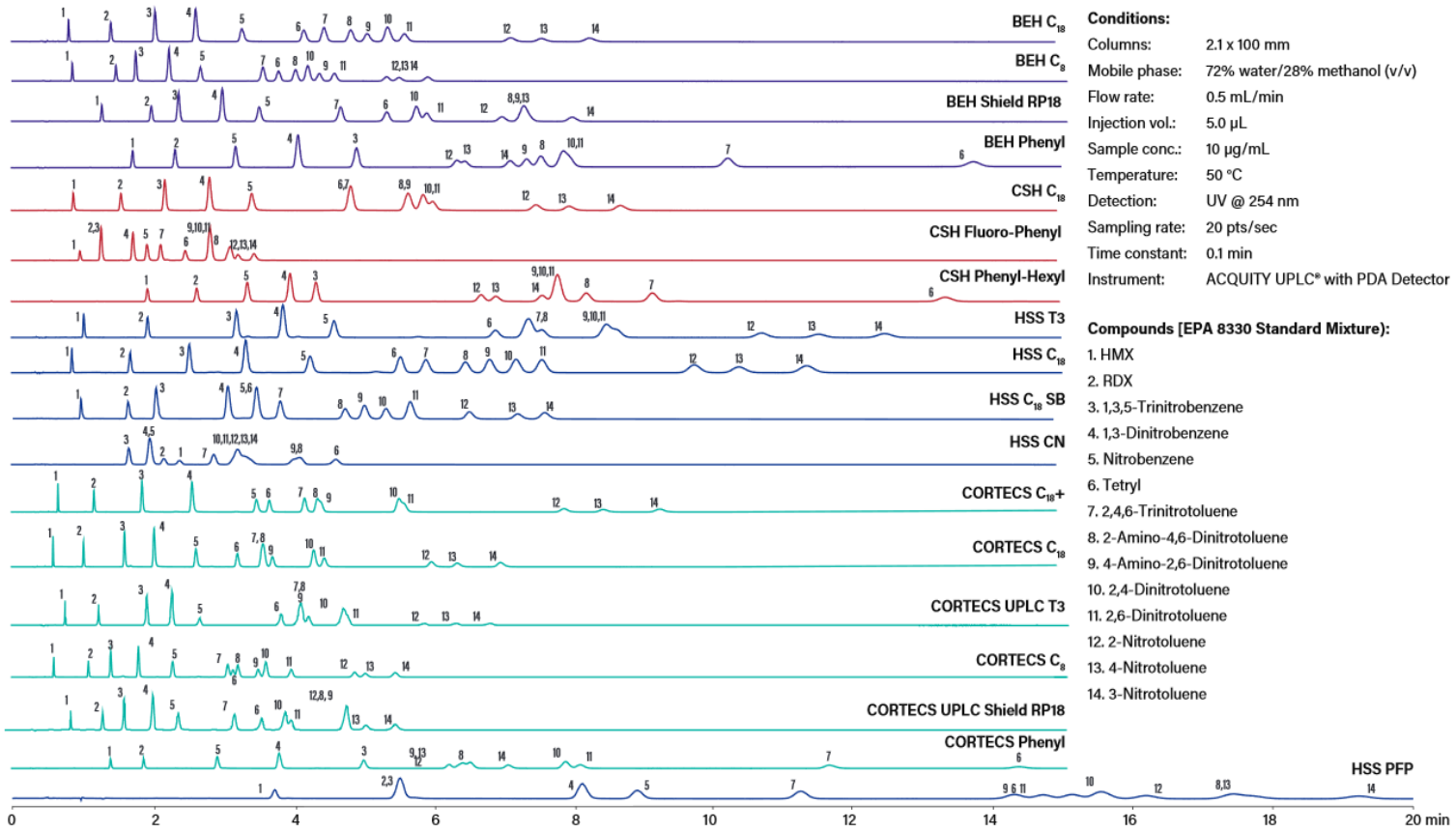
Selectivity Choices Nitroaromatic Compounds



Conditions:

Columns:	2.1 x 100 mm
Mobile phase:	72% water/28% methanol (v/v)
Flow rate:	0.5 mL/min
Injection vol.:	5.0 µL
Sample conc.:	10 µg/mL
Temperature:	50 °C
Detection:	UV @ 254 nm
Sampling rate:	20 pts/sec
Time constant:	0.1 min
Instrument:	ACQUITY UPLC® with PDA Detector

Compounds [EPA 8330 Standard Mixture):

1. HMX
2. RDX
3. 1,3,5-Trinitrobenzene
4. 1,3-Dinitrobenzene
5. Nitrobenzene
6. Tetryl
7. 2,4,6-Trinitrotoluene
8. 2-Amino-4,6-Dinitrotoluene
9. 4-Amino-2,6-Dinitrotoluene
10. 2,4-Dinitrotoluene
11. 2,6-Dinitrotoluene
12. 2-Nitrotoluene
13. 4-Nitrotoluene
14. 3-Nitrotoluene

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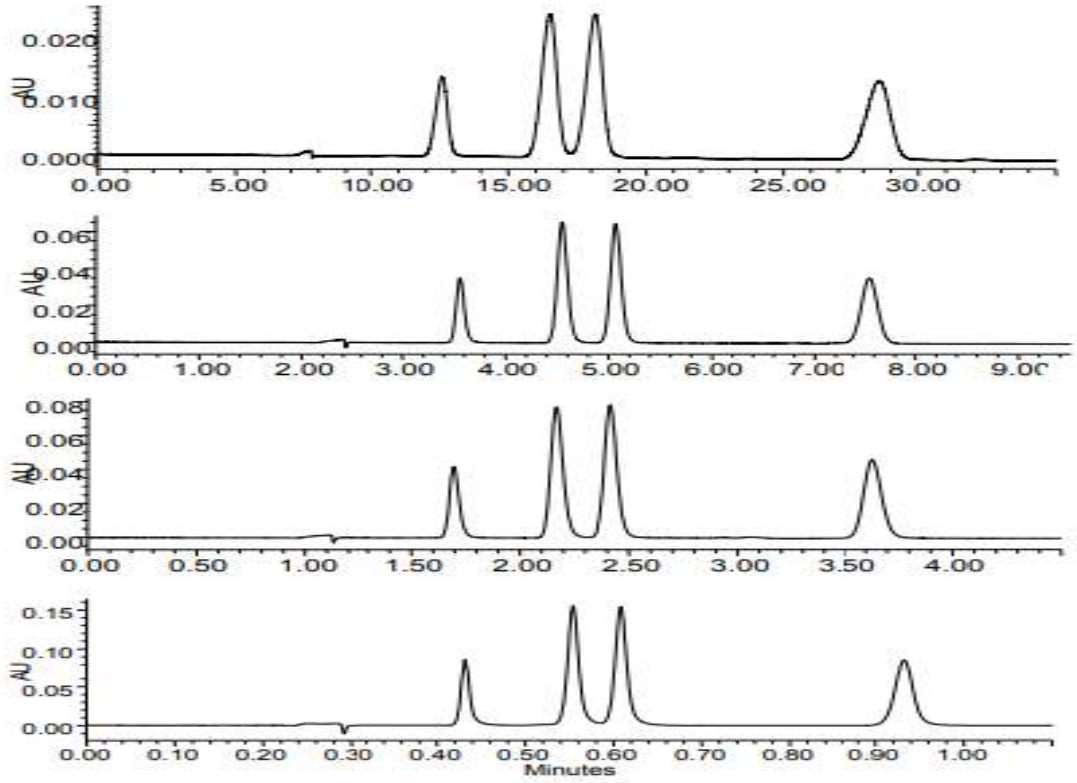
Optimized column dimensions to enhance efficiency



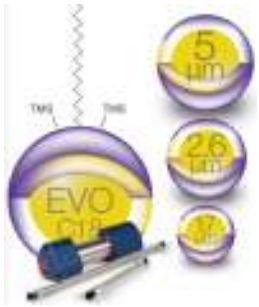
L/dp ~30,000

10 um – 250 mm
L/dp : 25,000

5 um – 150 mm
L/dp : 30,000

3.5 um – 100 mm
L/dp : 28,600

1.7 um – 50 mm
L/dp : 29,400



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Optimized column dimensions to enhance efficiency

Application Difficulty	Example	Suggested L/dp	Column Dimensions
Extremely Difficult	Complex matrix	> 85,000	1.7 um 150 mm : L/dp 88,235
Difficult	Impurity profile	> 50,000	5 um 250 mm : L/dp 50,000 3.5 um 150 mm : L/dp 42,857 2.5 um 150 mm : L/dp 60,000 1.7 um 100 mm : L/dp 58,823
Moderate Challenging	Related compound assay	> 30,000	5um 150 mm : L/dp 30,000 3.5 um 100 mm : L/dp 28,571
Easy	Few peak Well separated	> 15,000	5 um 75 mm : L/dp 15,000 3.5 um 50 mm : L/dp 14,285

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Column dimension

- Wider diameter typically leads to lower sensitivity, while narrow diameters correlate to higher sensitivity
- Wider columns require a larger sample with more solvent for analysis to work, while narrow columns can work with a smaller sample and less solvent as a mobile phase

Column Dimension (length x i.d. in mm)	Typical Flow Rates (mL/min)	Sample Amount(mg)	Sample Volume (µL)
150 x 1	0.06	≈ 0.05	0.05–1
250 x 2	0.25	≈ 0.2	0.2–10
250 x 3	0.6	≈ 1	1–20
250 x 4	1	≈ 5	5–80
250 x 10	6	≈ 30	30–500
250 x 25	39	≈ 200	200–3000

Merck : A practical guide to high performance liquid chromatography

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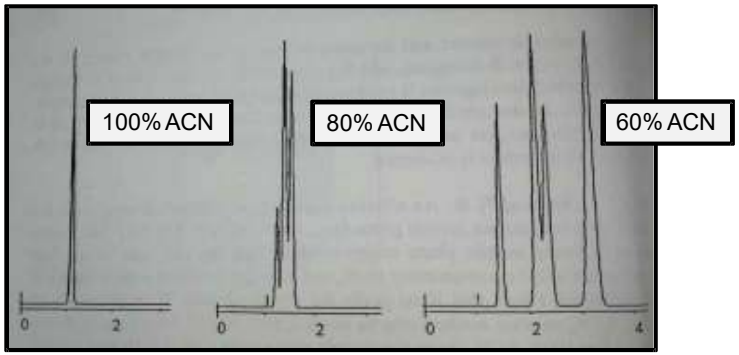
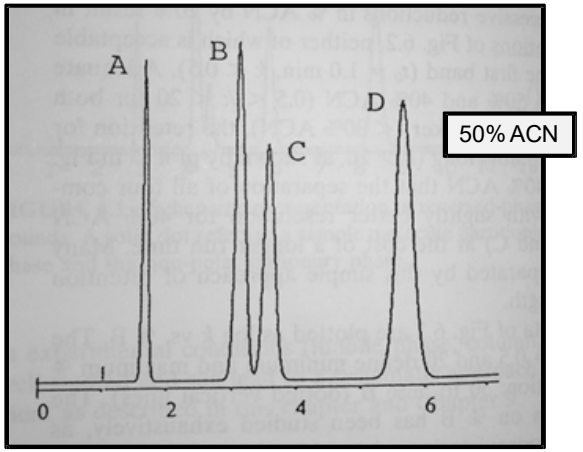
Retention in Reverse Phase Chromatography

- Mobile Phase Effects : Solvent strength depends on both the choice of organic solvent an its concentration in mobile phase





Effect of a change in percent organic on RPC separation of a hypothetical sample

Condition : 15x 0.46-cm C18 column ,1.5 ml/min flow rate

Retention in Reverse Phase Chromatography

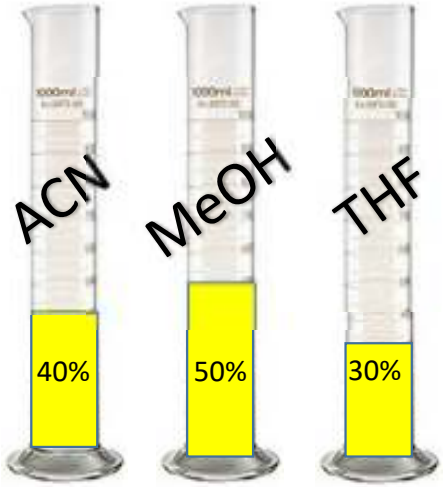
Solvent Strength : Three commonly used RPC solvents

- Methanol
- Acetonitrile
- Tetrahydrofuran

Solvent strength

For example : % of organic solvent in mobile phase having the same strength (giving similar values of k')

- 40% ACN : 50% MeOH : 30% THF



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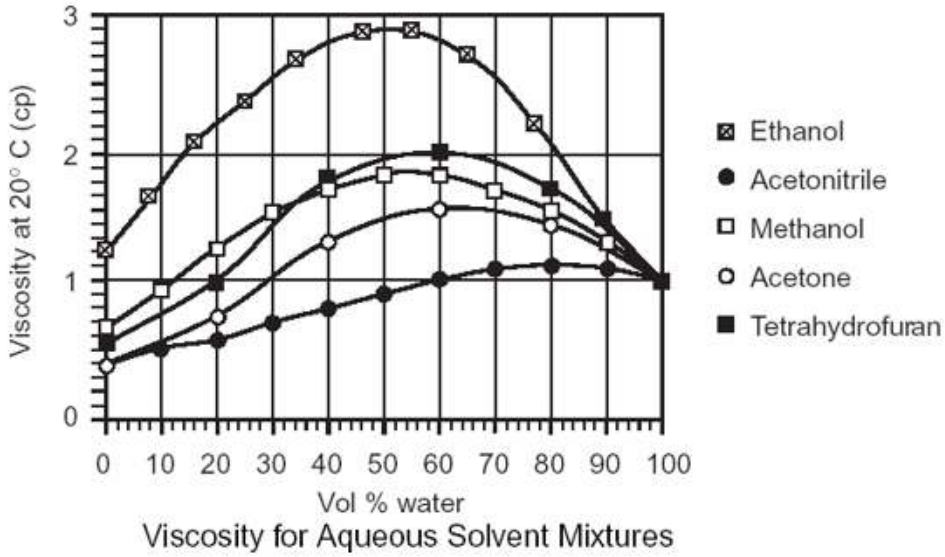
Reverse phase solvent considerations

- Methanol
  - High viscosity when mixed with water
  - Less expensive than acetonitrile
  - High UV cutoff than acetonitrile
- Acetonitrile
  - Low UV cutoff (190 nm)
  - High elution strength than methanol
  - High cost
- Tetrahydrofuran
  - Strong elution strength
  - Strong solubilizing power, highly toxic
  - High UV cutoff (212 nm)



Viscosity for Aqueous Solvent Mixtures

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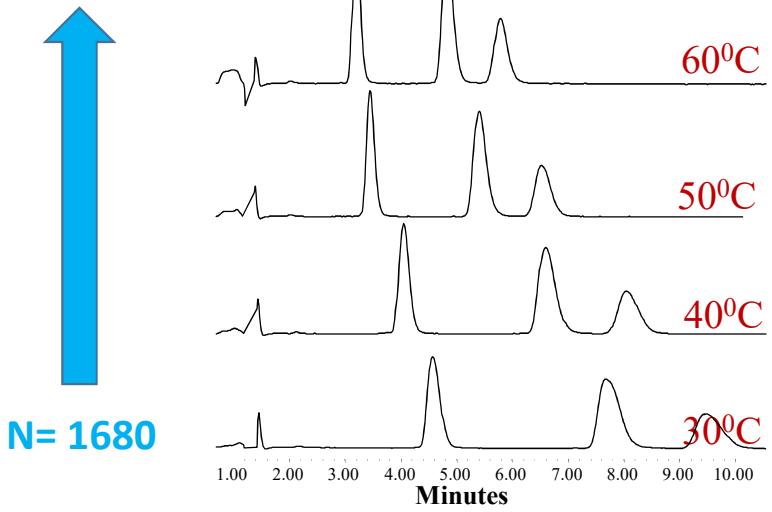
Retention in Reverse Phase Chromatography

Column and Temperature Effects :

- An increase in temperature by 1 ^{0}C will usually decrease value of ***k***' by 1 – 2 % for non-ionic compounds

Plates

N= 2250



N= 1680

Back Pressure

1160 psi

1920 psi

Benefits of temperature in HPLC :

* Shorter Run Time
* Better Sensitivity
* Sharper Peaks, N
* Lower Back Pressure (less viscosity)

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Wavelength Cutoffs of Common Solvents

UV cutoff: the wavelength at which the solvent absorbance in a 1 cm path length cell is equal to 1 AU (absorbance unit) using water in the reference cell.

Solvent	*UV Cutoff*	*Solvent*	*UV Cutoff*
n-Pentane	190	Methyl-isobutyl-ketone	334
Isooctane	215	Tetrahydrofuran	230
Petroleum ether	210	Ethylene dichloride	230
Cyclohexane	200	Methyl ethyl ketone	330
Cyclopentane	200	Acetone	330
Carbon disulfide	380	Dioxane	215
Carbon tetrachloride	265	Ethyl acetate	256
Amyl chloride	225	Methyl acetate	260
Xylene	290	Amyl alcohol	210
Isopropyl ether	220	Diethyl amine	275
Toluene	285	Nitromethane	380
n-Propyl chloride	225	Acetonitrile	190
Benzene	280	Pyridine	330
Ethyl ether	220	2-Butoxyethanol	220
Ethyl sulfide	290	Isopropanol	205
Chloroform	245	n-Propanol	210
Methylene chloride	233	Ethanol	210
Methanol	205	Ethylene glycol	210

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Wavelength Cutoffs of Common Mobile Phase

Mobile Phase	UV Cutoff	Mobile Phase	UV Cutoff
Acetic acid, 1%	230	Tween™20,0.1%	190
Trifluoroacetic acid, 0.1%	205	Hydrochloric acid, 0.1%	190
Triethylamine, 1%	235	Diammonium Phosphate, 50mM	205
PIC B-6, 1 vial/liter	225	PIC A, 1 vial/liter	<200
PIC D-4, 1 vial/liter	190	PIC B-6(low uv), 1 vial/liter	190
Ammonium Bicarbonate,10mM	190	Ammonium acetate, 10mM	205
HEPES, 10mM, pH 7.6	225	EDTA, disodium, 1mM	190
Potassium Phosphate,		MES, 10mM, pH 6.0	225
monobasic, 10mM	190	Sodium acetate, 10mM	205
dibasic, 10mM	190		
Sodium chloride. 1M	208	Sodium citrate, 10mM	225
Sodium formate, 10mM	200	TRIS HCl, 20mM, pH 7.0	204
BRIJ 35, 0.1%	190	pH 8.0	212
Sodium dodecyl sulfate, 0.1%	190	CHAPS, 0.1%	215
		Triton-X™100, 0.1%	240

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pH modifier and buffer in reverse phase chromatography

- pH of mobile phase can effect on the retention of ionizable (acidic or basic) analytes
- Commonly, the concentration should be kept in the 5 to 100 mM ,

  use the lowest concentration that gives reproducible results
- Be carefully to avoid buffer precipitation at higher concentrations in the organic solvent
- Hydrolysis of stationary phase at low pH and dissolution of silica at high pH

Common buffers in reverse phase chromatography

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Buffer	pKa	UV cutoff (nm)
Trifluoroacetic acid	0.3	210
Phosphate	2.1,7.2,12.3	190
Citrate	3.1, 4.7, 5.4	225
Formate	3.8	210
Acetate	4.8	210
Carbonate	6.4,10.3	200
Tris(hydroxymethyl) aminomethane	8.3	210
Ammonia	9.2	200
Borate	9.2	190
Diethylamine	10.5	235

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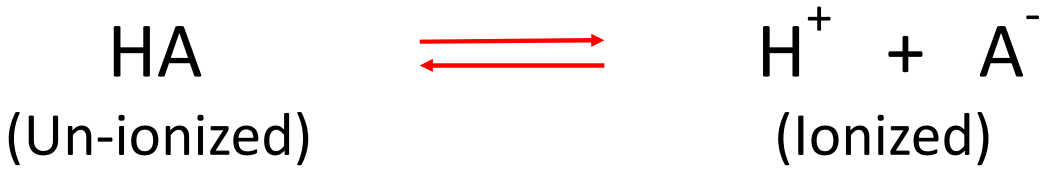
Ion Suppression Acids

Ionization of acids and bases dissociation of molecule

Acids



- More hydrophobic
- More retained

50% ← @ pKa → 50%

100% ← Low pH → 0%

0% High pH → 100%

- Less hydrophobic
- Less retained

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Ion Suppression Bases

Ionization of acids and bases dissociation of molecule

Bases

- More hydrophobic
- More retained

50% ← @ pKa → 50%
0% Low pH → 100%
100% ← High pH 0%

- Less hydrophobic
- Less retained

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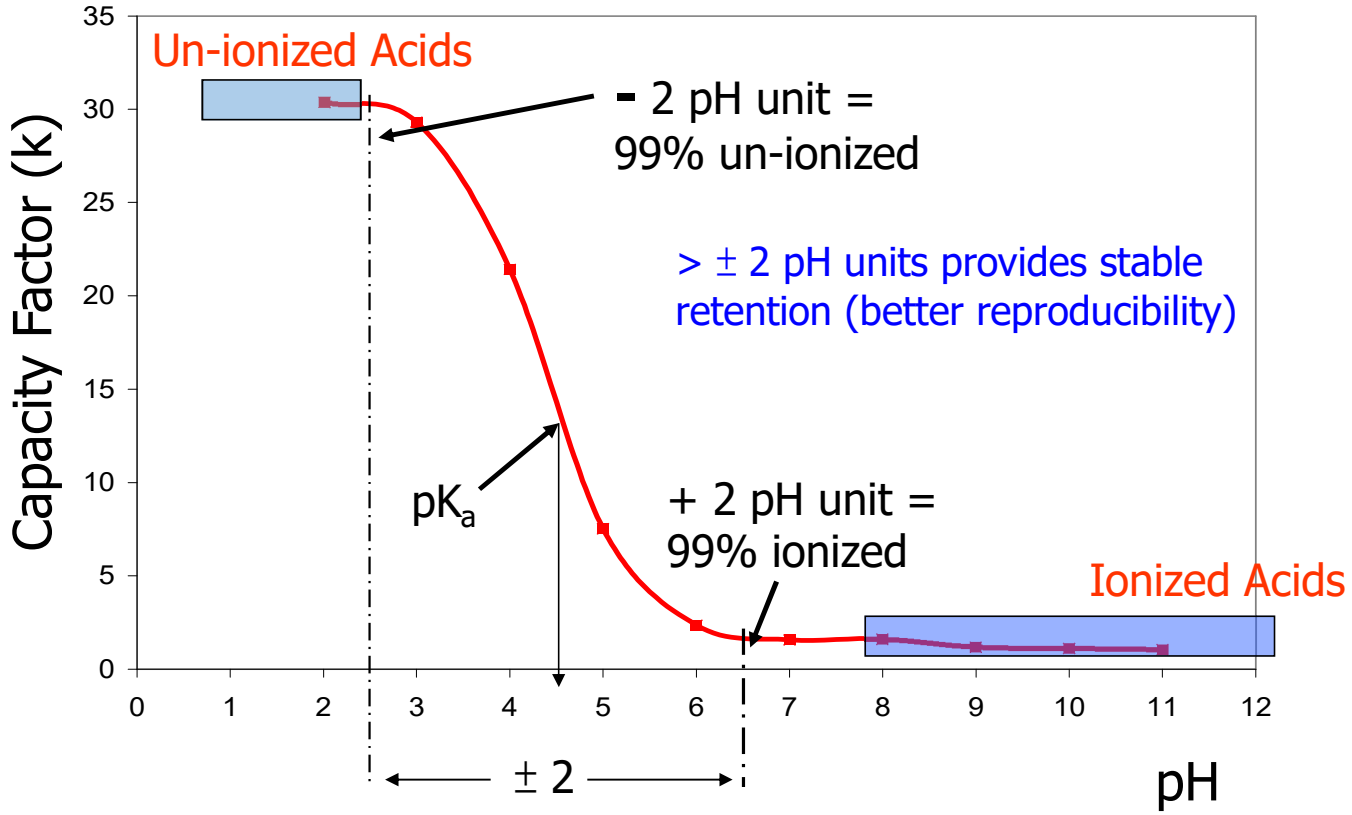
More Robust Methods—Stable Zones



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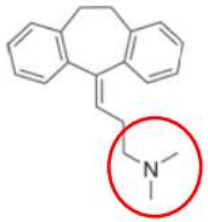
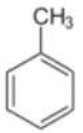
Effect of pH on analyte retention

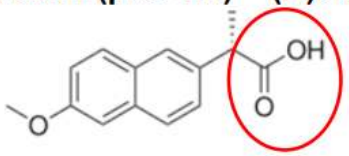
Amitriptyline (pKa 9.4) = (B)ase

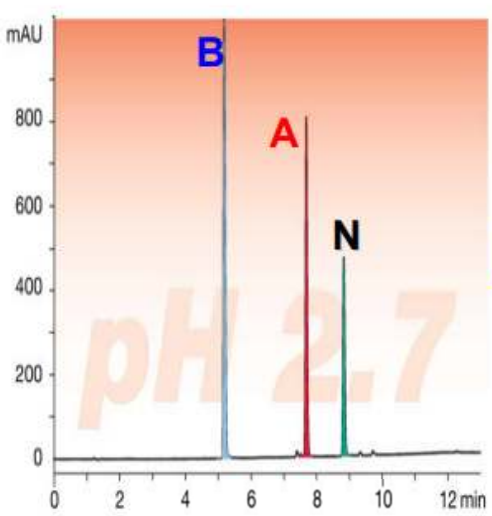


Toluene = (N)eutral

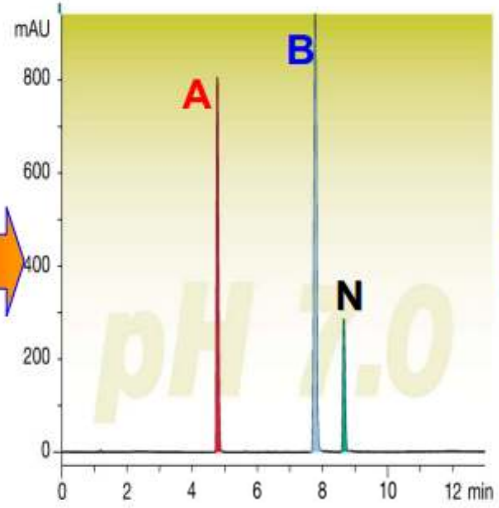


Naproxen (pKa 4.5) = (A)cid

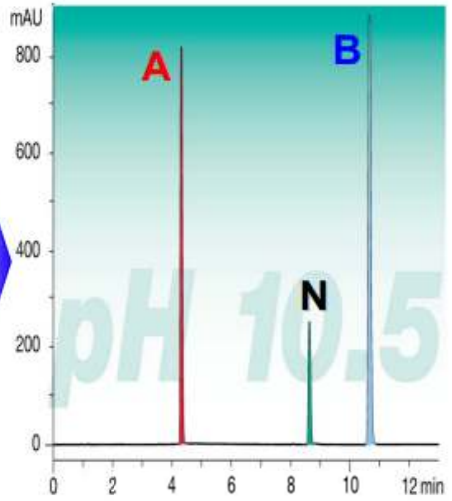




pH 2.7



pH 7.0



pH 10.5

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Paired Ion Chromatography

- Typical reversed phase chromatography is obtained for ionic molecules
- Adding PIC reagents causes increased retention for ions of opposite charge and decreased retention for ions of the same charge as the PIC reagent
- Increasing the PIC reagent lipophilicity causes increased retention of paired ions
- Increasing PIC reagent concentration increases retention of paired ions up to a limiting value

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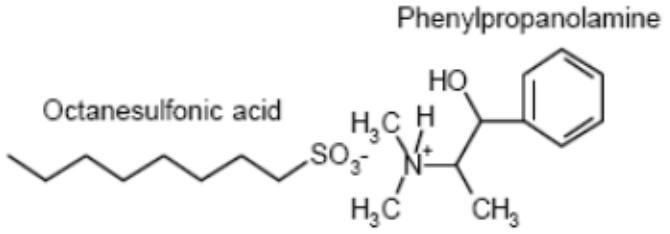
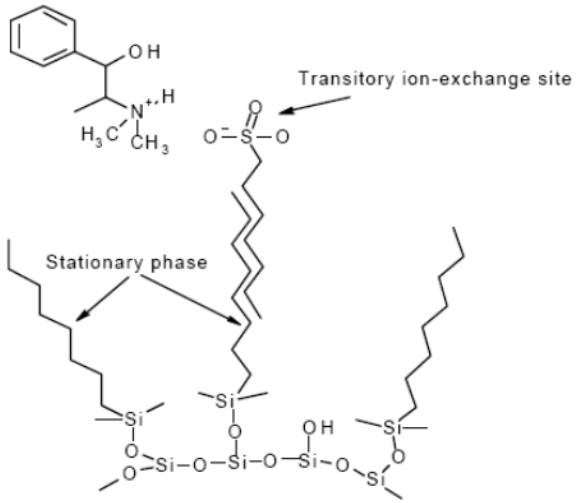
Interaction mechanisms

- Ion-pairing agents : interacting with any counter-ions in solution, thus forming pseudo-neutral complexes



- Transitory ion-exchange site : the hydrophobic portions of the ion-pairing agents partitioning into the alkyl bonded phase of the column, forming transitory ion-exchange sites



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Ion Pairing for Reverse Phase Separations

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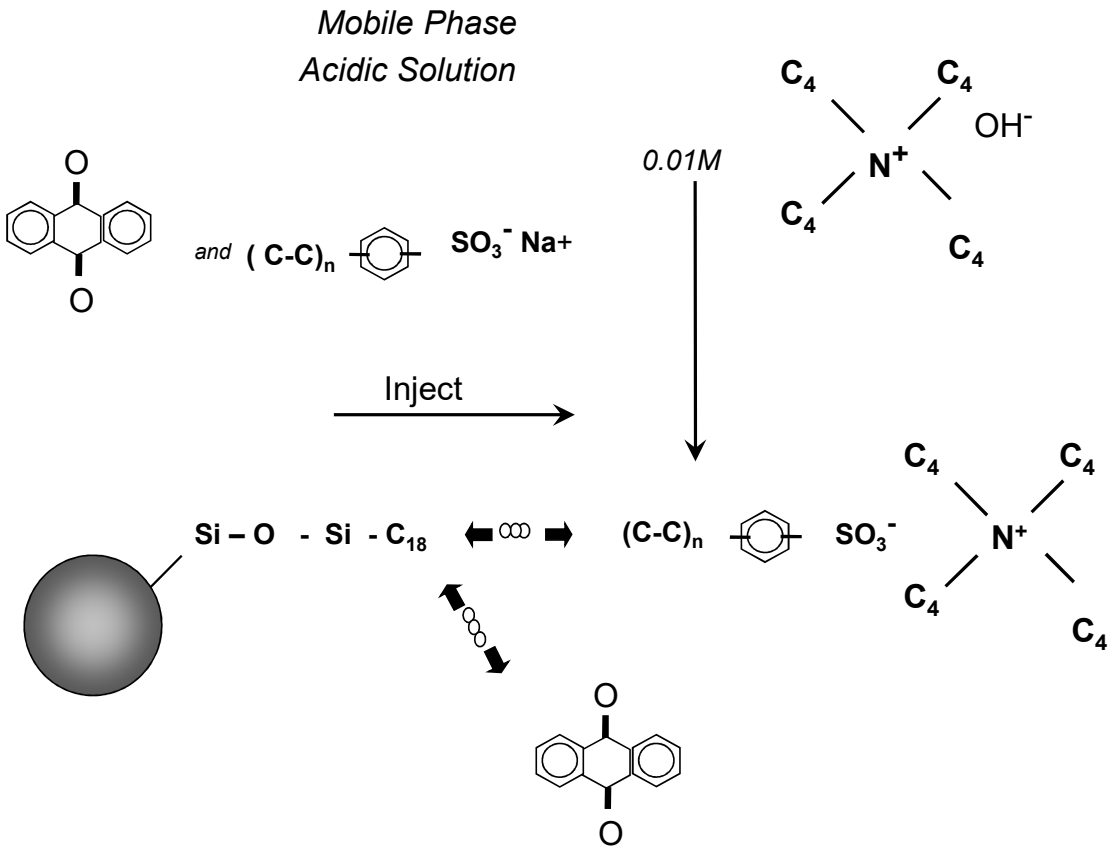


Advantages and Disadvantages of paired ion Chromatography

Advantages :

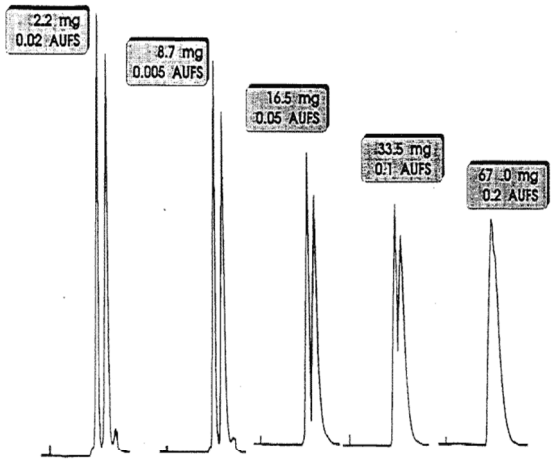
- Can separate both ionized and non-ionized analytes simultaneously
- Improve peak shape
- High reproducibility of results

Disadvantages :

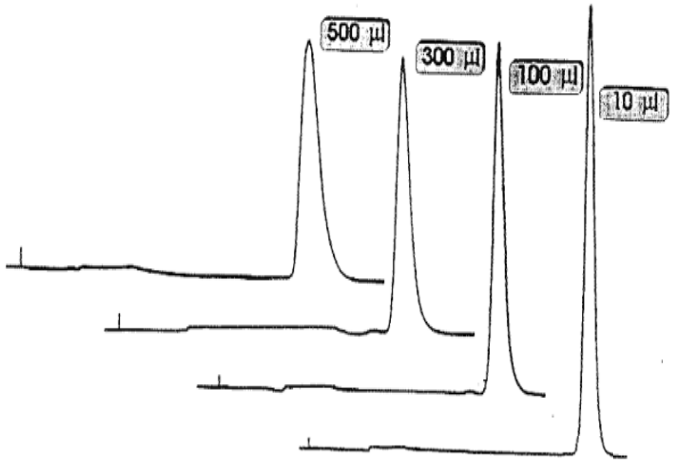
- Column equilibration takes longer time (20-50 column volumes or more)
- High cost of ion pair reagents

Sample injection, mass and volume overload

- An increase of the volume or mass of a sample injected onto a column has a positive influence on the sensitivity of an analysis, However this approach is limited, as at a certain point both mass and/ or volume overload can be observed in a chromatographic separation



Mass overload



Volume overload

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Common HPLC Detectors

Detector	Response	Sensitivity
Refractive index (RI)	Universal	μg
UV/Vis absorbance (UV/Vis)	Specific : Chromophore	ng
Fluorescence (FLD)	Specific : Fluorophore	pg
Electrochemical (ECD)	Specific : Redox	pg
Conductivity	Specific : Ion	pg
Evaporative light scattering (ELSD)	Near universal	ng
Mass spectrometry (MS)	Specific	ng

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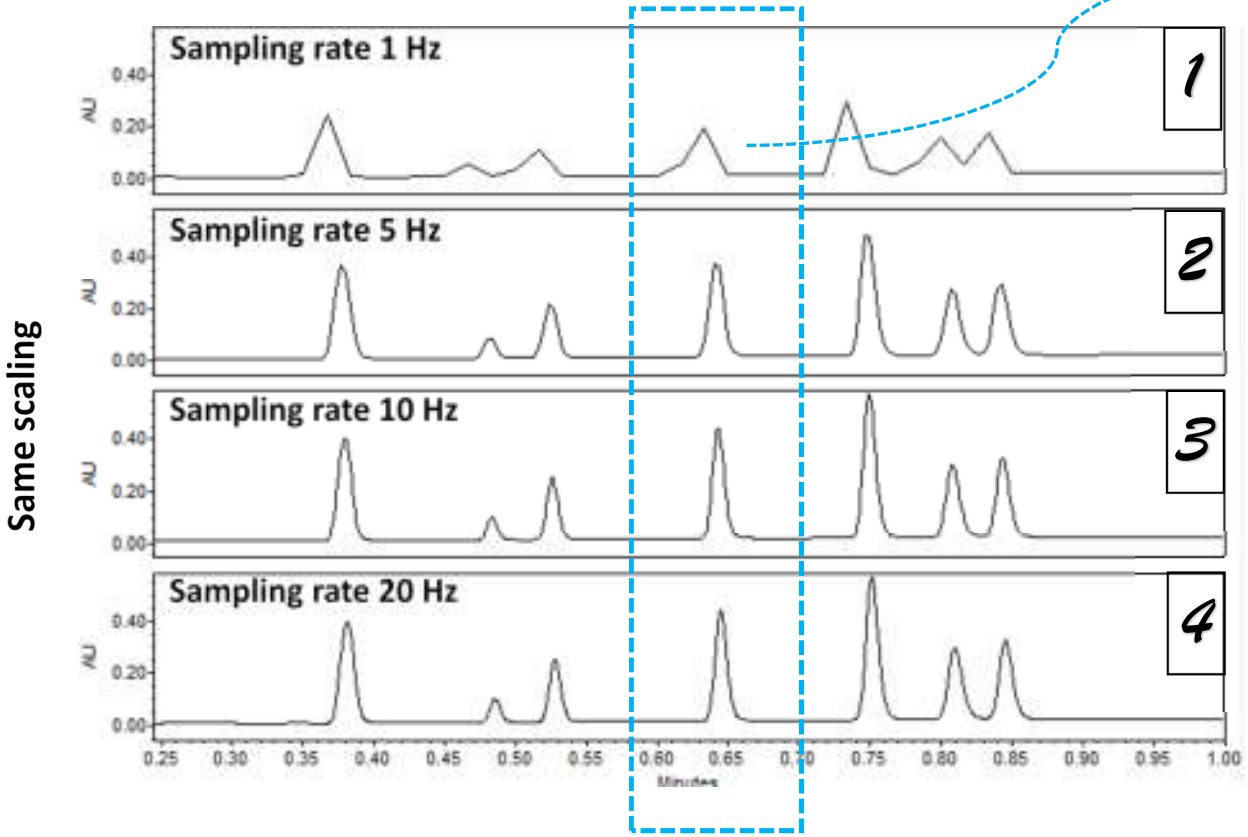
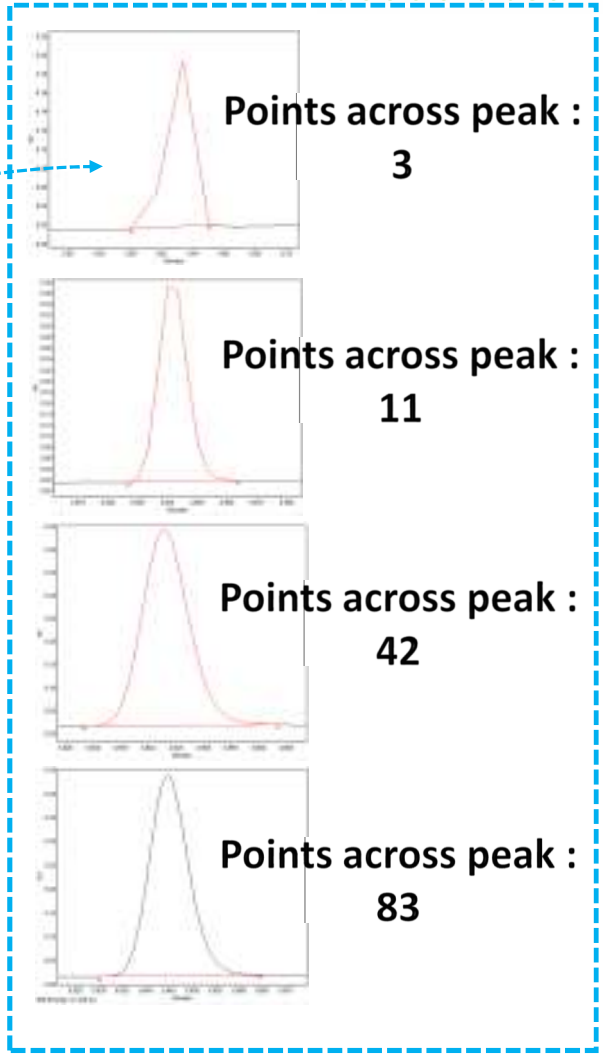
Detector setting : Sampling rate

- Typically, need to acquire at *least 15 to 20 points* across a chromatographic peak for good quantitation





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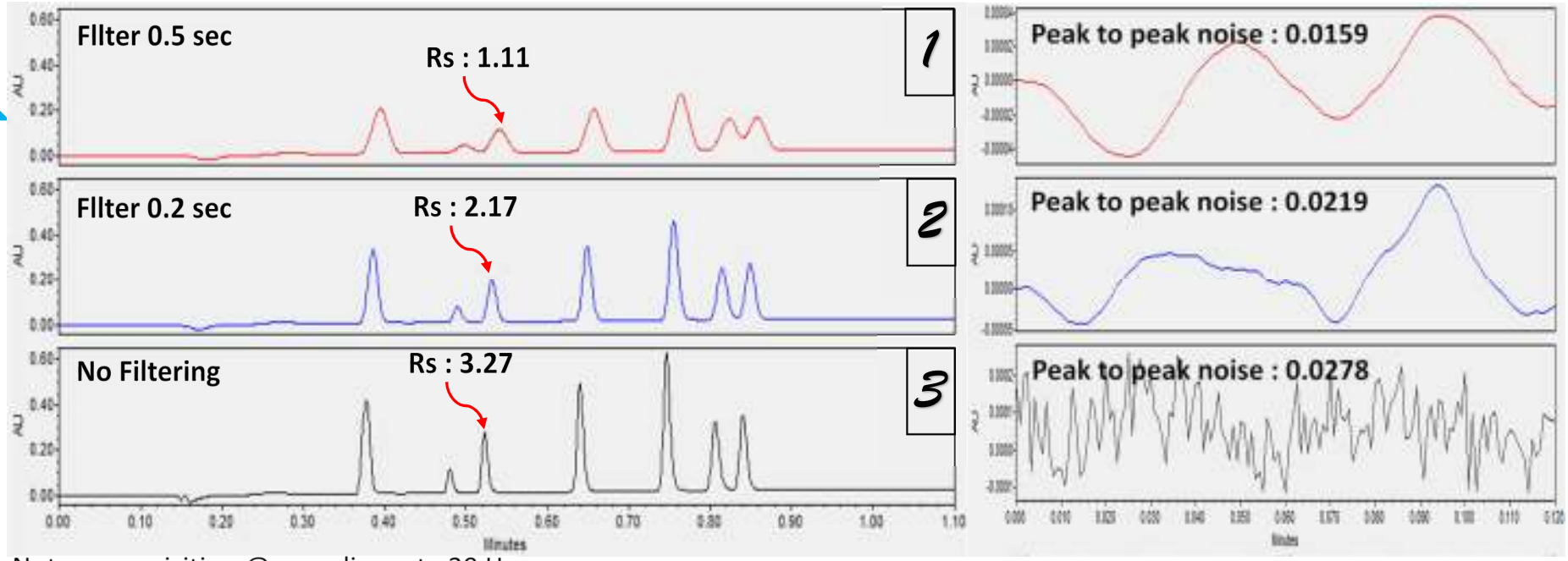
Detector setting : Filter time constant

- Filter time constant is a mathematical algorithm applied to a data set that smoothes out higher frequency noise



Filtering Time Constant

Notes : acquisition @ sampling rate 20 Hz

Filtering Time Constant ↑ Baseline noise ↓ Resolution ↓

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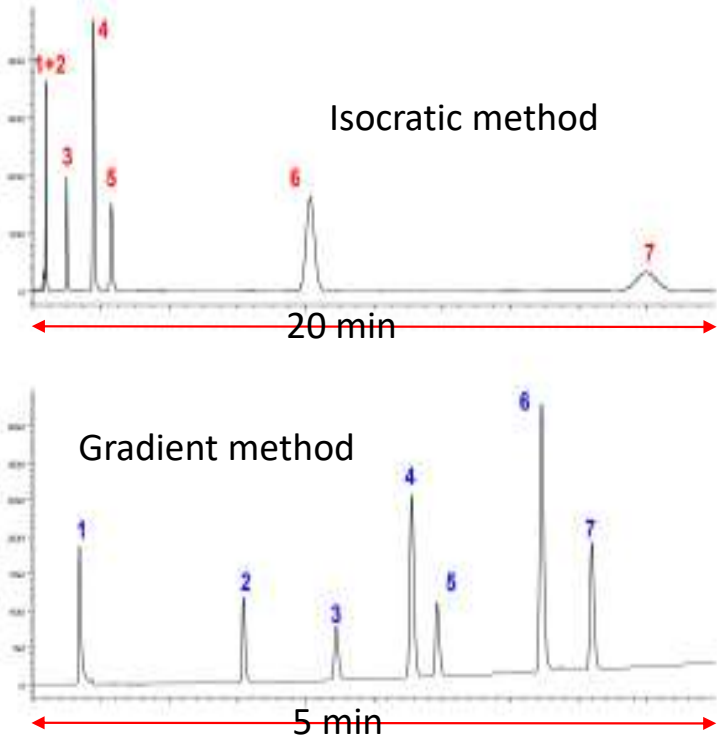
Gradient analysis

- Separation samples with wide polarity range
- Gives chromatograms with sharp peaks throughout
- Reduction of the total run time of separations complex samples
- Cleaning or regeneration of the chromatographic column
- Need to adequately re-equilibrate the column



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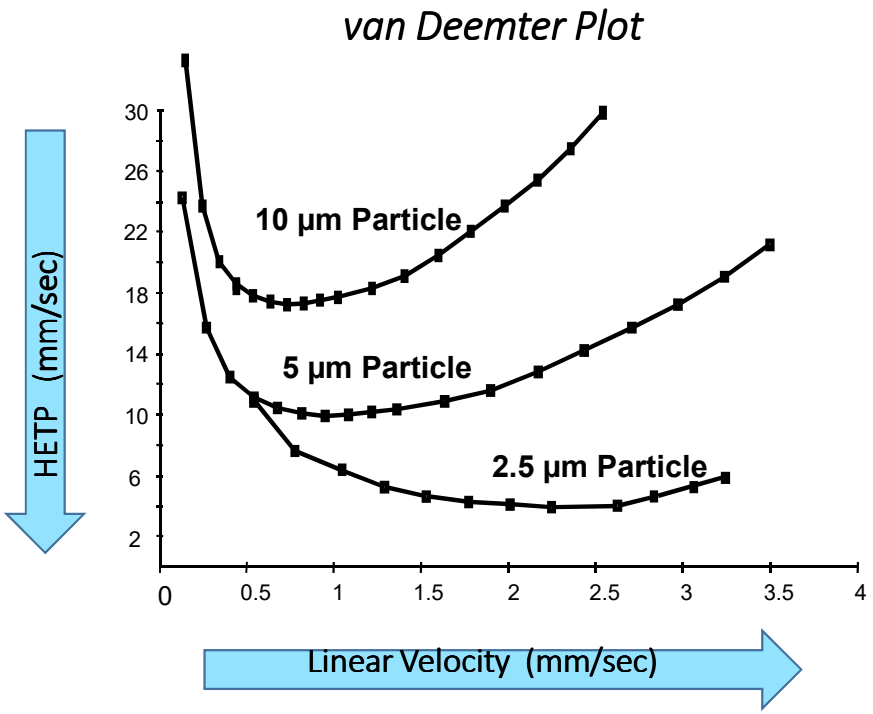
Flow rate optimization



- Flow rate affect analyte retention time
- Slow linear velocity can result in overlapping **peaks**, too fast a velocity can reducing resolution.
- flow rate change in gradient elution can more affect than isocratic elution

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Typical flow rate, backpressure

- Typical flow rate dependent of particle size & column id

Particle Size (µm)	Internal Diameter (mm)	Typical Flow (mL/min)	Typical Pressure (PSI)		
			50 mm**	150 mm**	250 mm**
1.3	2.1	0.5	10500	—	—
1.7	1.0	0.1	2600	—	—
1.7	2.1	0.5	5300	7300	—
1.7	3.0	0.3	7000	—	—
2.6	1.0	0.1	1700	4300	—
2.6	2.1	0.5	3000	6800	—
2.6	3.0	0.8	2300	5900	—
2.6	4.6	1.8	2800	5300	6900
3.5	4.6	1.5	—	2600	—
5	2.1	0.3	580	1200	—
5	3.0	0.5	620	1050	—
5	4.6	1.3	1100	1600	1700
5	10	5	—	2100	3000
5	21.2	25	630	1300	2300
5	30	50	670	1600	2300
5	50	80	—	1000	—

** Column Length

Web : www.phenomenex.com

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Case study

Initial purity method development of an NCE using a broad gradient

(HPLC AND UHPLC FOR PRACTICING SCIENTSTS 2019)

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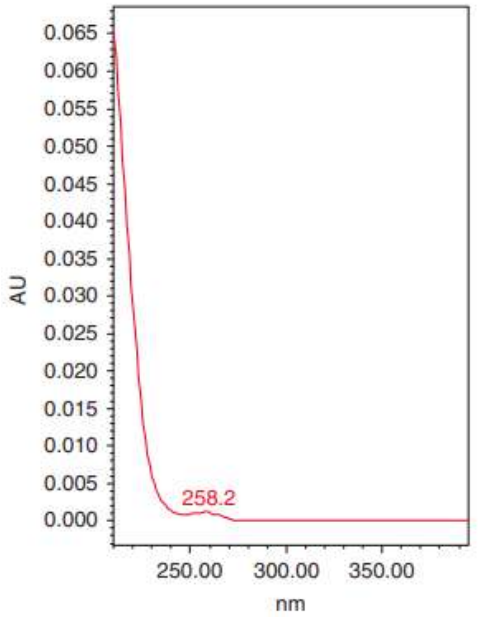
NCE : Method Development

1. Define method and separation goals
   - Method goal : MS-compatible stability-indicating method
2. Gather sample and analyte information
   - The API is a basic salt with a pKa of ~10
   - Good solubility in water
   - The selection of λmax at 258 nm is an issue due to low sensitivity



UV spectrum of NCE

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NCE : Method Development (Cont.)

3. Initial method development

- Using high-purity silica-based C18 column (100 × 3.0 mm, 3 µm)
- Broad gradient of 5–95%B in 15 minutes
  - MPA = 0.1% formic acid in water, MPB = 0.1% formic acid in acetonitrile
- API sample was dissolved in a 50/50 acetonitrile/water diluent (1.0 mg/ml)
- PDA detector was used to collect data between 210 and 400 nm & various wavelengths of 260, 230, 220, and 210 nm

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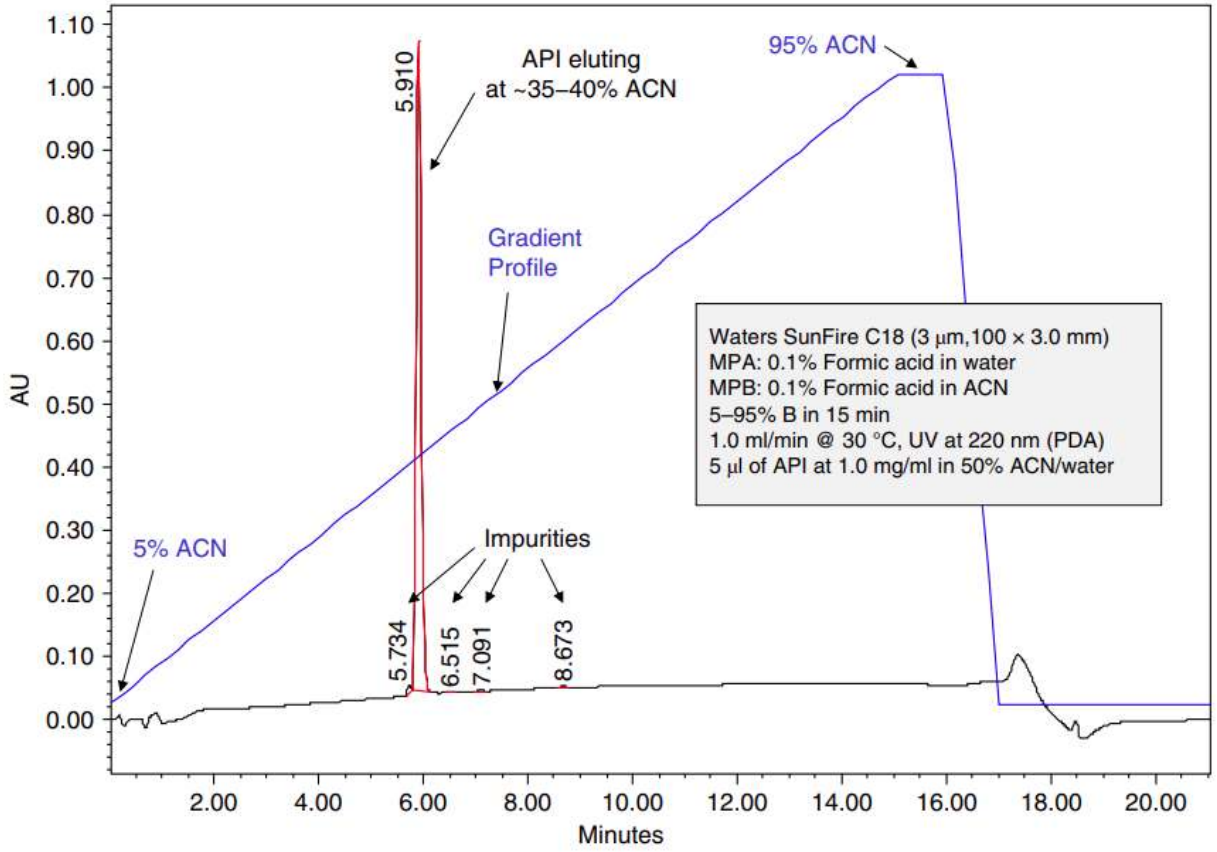
The first chromatogram of NCE development



- The API elutes at %B of 35–40% of acetonitrile, showing the presence of several impurities between five and nine minutes

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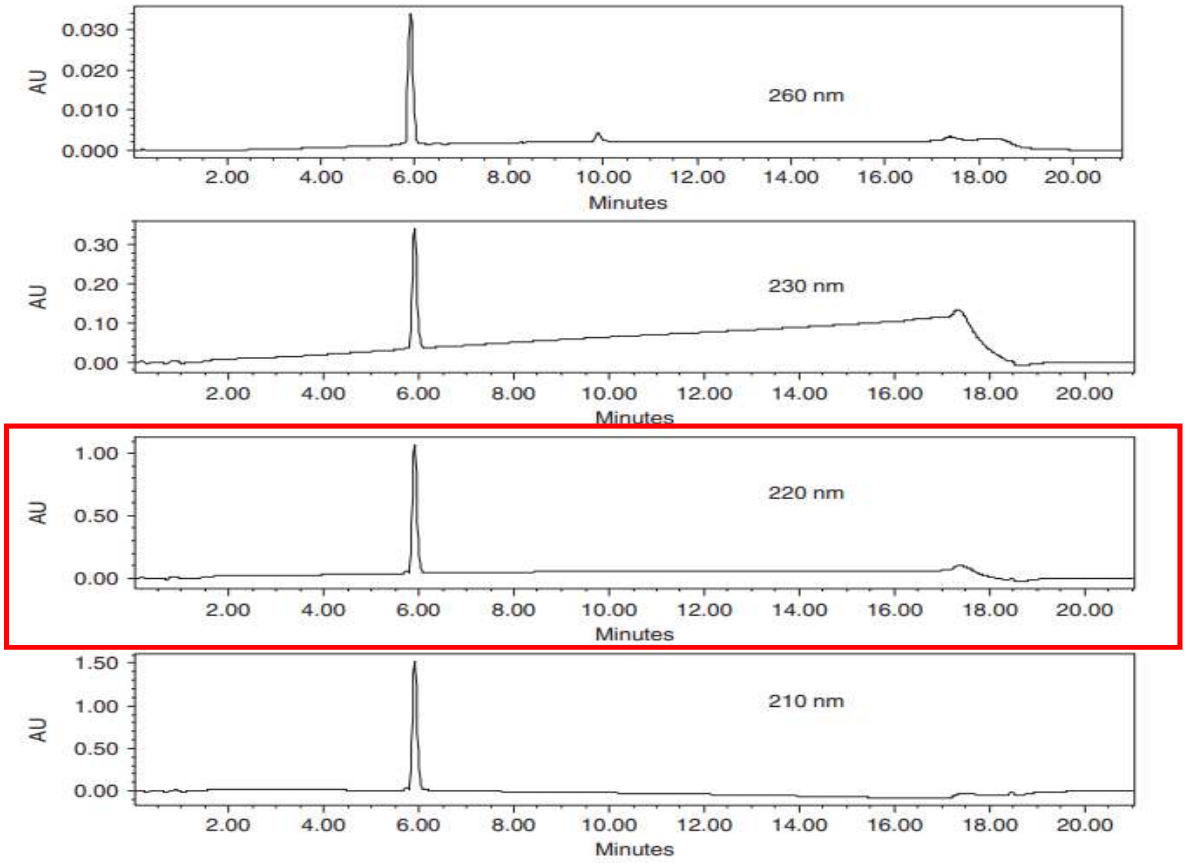
NCE chromatograms to be plotted at various wavelengths



- A detection wavelength of 220 nm produced a strong signal for the API at ~1 AU with an acceptable gradient shift of the baseline

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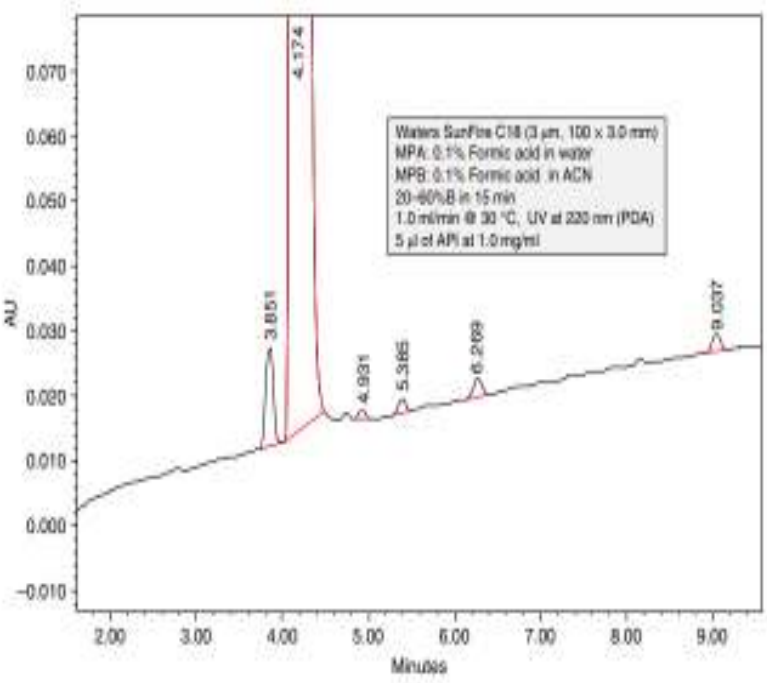
NCE : Method Development (Cont.)

4. Method fine-tuning and optimization (adjust gradient profile)

- The next scouting gradient was reduced to a narrower range of 20–60% ACN yielding a chromatogram with enhanced resolution of the impurities



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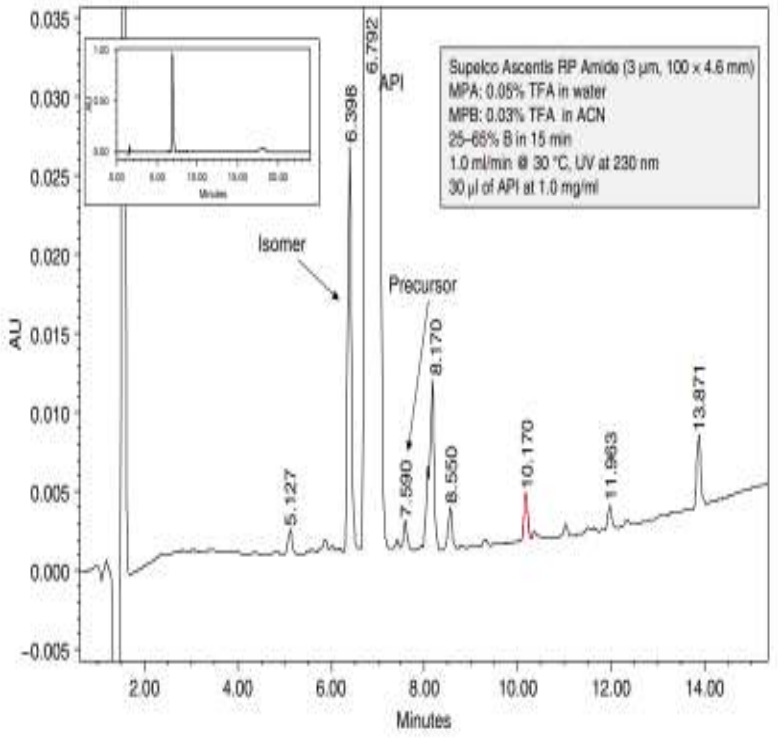
NCE : Method Development (Cont.)

5. Method fine-tuning and optimization (column type/mobile phase)

- Switching the MP from 0.1% formic acid to 0.1%TFA yielded higher retention and provided a partial separation
- Two polar-embedded columns (Waters XTerra RP18 and Supelco Discovery RP-amide) were evaluated
- Reducing the level of TFA in MP for deduce baseline shift at 230 nm and adequate sensitivity (LOQ = 0.05%)



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Questions ?

Thank you